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Net export of *E. coli* from the Toenepi wetland cannot be explained by growth of naturalized *E. coli* in the water column

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Abstract: Runoff from agricultural land is recognized as an important source of contaminants—nitrogen, phosphorus, sediment and *Escherichia coli*—that impact water quality. Constructed wetlands have been promoted as a green infrastructure approach to attenuate these contaminants. A considerable amount of work has been carried out on nitrogen, phosphorus and sediment attenuation through wetlands and these processes are reasonably well understood. There has been much less research on the attenuation of faecal microbes, such as *E. coli*, through wetlands. A research/demonstration constructed wetland was established in the Toenepi catchment, Waikato, New Zealand (NZ) to investigate nutrient removal from sub-surface drainage from grazed dairy pasture. As a part of the earlier nutrient monitoring of the Toenepi wetland some samples were analyzed for *E. coli* concentrations. The surprising result of the *E. coli* testing was the frequently higher concentrations of *E. coli* in the outflow than the inflow to the wetland, indicating a "net export" of *E. coli* from this wetland. This apparent net export of *E. coli* from the Toenepi wetland led to a hypothesis that some *E. coli* strains were becoming naturalized and hence growing in the wetland. "Naturalized *E. coli*" is a term used to describe subtypes of *Escherichia* that persist/grow in aquatic environments, and hence, do not represent "recent faecal contamination". A recent genome study identified potentially naturalized strains of faecal *E. coli* and non-faecal *E. coli*-like *Escherichia* in the Toenepi wetland. *E. coli* isolates from fresh and aged faecal inputs and a naturalized *Escherichia* strain isolated from the Toenepi wetland were used in a series of microcosm (in the lab) and mesocosm (placed in the wetland) studies to assess if the isolates could grow in the water and if so, determine their potential growth rate. In this study, a model of the putative *E. coli* concentrations in the water flowing into and out of the 2-cell wetland system was developed based on a 15-minute time step and assuming first order growth rates in the water column. Modelled water flows were based on measured inflow rates over the winter of 2006 and model outputs compared to the *E. coli* concentrations measured during that winter flow period. The mean and maximum growth rates measured for any of the *E. coli* isolates were 0.1 and 0.2 (*ln* day^{-1}), respectively. When these growth rates were used in the model for outlet water concentrations, the modelled *E. coli* concentrations in the outlet were always less than the average measured outlet *E. coli* concentrations. To fit the model to measured outlet concentrations required increasing the growth rate to 0.3 day^{-1}. This high *E. coli* grow rate appears to indicate that *E. coli* growth in the water column alone cannot explain the net export of *E. coli* from the Toenepi wetland system. Furthermore, the model predicted that the *E. coli* concentrations in the outflow would progressively increase during the low flow period between major flow events and then decrease as the wetland water was effectively diluted with low *E. coli* concentrations in the storm inflow. Detailed examination of the measured *E. coli* concentrations showed that the outflow concentrations did not increase during the low flow periods but increased dramatically at the beginning of the high flow periods and often even before the flow rate in the wetland increased. This dynamic response in the wetland further confirms that *E. coli* growth in the water column cannot explain the net export of *E. coli* from this wetland complex. The rapid increase in *E. coli* concentrations in the outflow, coinciding with or preceding the increase in flow rates, indicates that the source of *E. coli* may be attached to the surfaces of the plants or leaf litter in the wetland. This new hypothesis will require further investigation.

Keywords: *Escherichia coli, agriculture, water quality, attenuation, green infrastructure*

1. INTRODUCTION

Runoff from agricultural land is recognized as an important source of contaminants—nitrogen, phosphorus, sediment and *Escherichia coli*—that impact on water quality. Constructed wetlands have been promoted as a green infrastructure approach to attenuate these contaminants (Asare et al. 2022). A considerable amount of work has been carried out on nitrogen, phosphorus and sediment attenuation through wetlands and these processes are reasonably well understood (Shukla et al. 2021). There has been much less research on the attenuation of faecal microbes, such as *E. coli*, through wetlands. Research has shown *E. coli* concentrations generally decreasing through a wetland although outlet concentrations can remain relatively consistent indicating some microbial persistence (Hathaway et al. 2011). A research/demonstration constructed wetland was established in the Toenepi catchment, Waikato, New Zealand (NZ) to investigate nutrient removal from sub-surface drainage from grazed dairy pasture (Tanner et al. 2005). The Toenepi is an intensively farmed catchment with silt loams on the valley bottoms, yellow brown loams on the lower slopes and a clay loam on the upper slopes and has an annual rainfall of 1132mm (Wilcock et al. 1999). As a part of the earlier nutrient monitoring of the Toenepi wetland some samples were analyzed for *E. coli* concentrations. The surprising result of the *E. coli* testing was the frequently higher concentrations of *E. coli* in the outflow than the inflow to the wetland, indicating a "net export" of *E. coli* from the wetland (Stott et al. 2014). Note that other monitored wetlands in NZ and internationally, generally show decreasing *E. coli* concentrations through wetlands (Hathaway et al. 2011; Mulling et al. 2013). This apparent net export of *E. coli* from the Toenepi wetland led to a hypothesis that some *E. coli* strains were becoming naturalized and hence growing in the wetland.

E. coli is used in water quality monitoring as a faecal indicator organism that indicates recent faecal contamination and, by association, the risk of disease organisms also being present in the water. "Naturalized *E. coli*", is a term used to describe an *E. coli*-like bacteria that returns a positive result in the typical water quality test for *E. coli* but is actually adapted to survive or grow in the environment, and hence, does not represent "recent fecal contamination" (Devane et al. 2020). Some early investigations of the Toenepi wetland indicated some evidence of clonal strains of *E. coli* present (Jennings 2008; Perchec-Merien & Lewis 2012). A recent genome study identified potentially naturalized strains of faecal *E. coli* and non-faecal *E. coli*-like *Escherichia* (cryptic clades) in the Toenepi wetland (Cookson et al. 2022). *E. coli* isolates from fresh and aged faecal inputs and a cryptic clade *Escherichia* strain isolated from the Toenepi wetland were used in a series of microcosm (in the lab) and mesocosm (placed in the wetland) studies to assess if the isolates could grow in the water and if so, determine their potential growth rate.

The aim of this work was to develop a simple wetland model to test the hypothesis that the observed growth rates from the micro/mesocosm experiments could account for the apparent growth of *E. coli* observed in the Toenepi wetland.

2. METHODS

2.1. Model test period

As the wetland treats drainage water from agricultural land, the wetlands do not flow constantly. The wetlands usually dry out from November to March (Southern Hemisphere summer) but typically flow most of the winter drainage period with high flows during and shortly after rainfall events. The model test period was focused on the data collected during the wetland flows from late April to early October 2006. During this time water was continuously flowing through the wetland and there were 5 large flow events with the last 4 sampled over the high flow period. Event monitoring data were not delineated into base and stormflows for this modelling exercise and so *E. coli* concentrations during low flows were assumed to be similar to long-term low-flow samples collected from 2001 to 2005 (Stott et al. 2023).

2.2. Model assumptions and equations

The Toenepi wetland is composed of 2 shallow surface-flow rectangular cells in series. Each cell is 26 m long, 5 m wide and 0.3 to 0.6 m in depth with a volume of 65 m^3 per cell (Tanner et al. 2005). Tile drainage water flows into the inlet of Cell 1, then flows from the outlet of Cell 1 to Cell 2 before discharging from the outlet of Cell 2 into a small drain that eventually drains into the Toenepi Stream. Stott et al. (2023) observed smoothing of the flows through the wetlands as the individual cells increased in storage volume temporally during the beginning of an event before draining on the tail of the event. We have assumed steady-state flow with equivalent in and out flow volumes for each event throughout this period and hence the volume in each cell remained a constant 65 m^3. To seed the model, an initial load of *E. coli* in each cell was assumed to be 3.45 x 10^7 most probable number (MPN) calculated from 65 m^3 multiplied by 53 MPN $100mL^{-1}$, the long-term average low-flow concentrations into the wetland (Table 1). Each cell was assumed to be fully mixed and hence

the outputs were the average concentration in each cell. The model operated on a 15-minute timestep based on the measured flow data (L s^{-1}) provided by Stott et al. (2023).

The total number of *E. coli* in Cell 1 (*C1*) (MPN) at the end of each time step (*t*) was calculated using Equation 1.

$$C1_t = (C1_{t-1} \times e^k) + C1_{in,t} - C1_{out,t-1} \quad (1)$$

Where: *C1* is the number of *E. coli* in cell 1, *k* is the first order growth coefficient, $C1_{in}$ is the number of *E. coli* in the inflow to Cell 1, and $C1_{out}$ is the number of *E. coli* in the outflow from Cell 1.

$C1_{in}$ (MPN) per 15-minute timestep is calculated using Equation 2.

$$C1_{in} = F \times 15 \times 60 \times Ec_{in} \times 10 \quad (2)$$

Where: *F* is the flow rate (L s^{-1}), and Ec_{in} is the *E. coli* concentration in the inflow (MPN $100mL^{-1}$) from Table 1. The factors of 15 x 60 converts the flow rate from, L s^{-1} to L per 15-minute model timestep. The factor of 10 converts the *E. coli* concentration from MPN $100mL^{-1}$ to MPN L^{-1}.

$C1_{out}$ is calculated using Equation 3.

$$C1_{out} = (C1/V) \times F \times 15 \times 60 \quad (3)$$

Where: *V* is the volume of the cell (65,000 L).

The total number of *E. coli* in Cell 2 (MPN) at the end of each time step (*t*) was calculated using Equation 4.

$$C2_t = (C2_{t-1} \times e^k) + C1_{out,t} - C2_{out,t-1} \quad (4)$$

Where $C2_{out}$ (MPN) is the number of *E. coli* in the outflow from Cell 2.

$C2_{out}$ is calculated using Equation 5.

$$C2_{out} = (C2/V) \times F \times 15 \times 60 \quad (5)$$

The concentration of *E. coli* in the outflow from Cell 2 (MPN $100mL^{-1}$) was calculated using Equation 6.

$$Ec_{out} = C2 / (V \times 10) \quad (6)$$

Where: Ec_{out} is the concentration in the outflow (MPN $100mL^{-1}$).

2.3. Model inputs

Stott et al. (2023) provided the measured in- and out-flow rates into the wetland and accompanying *E. coli* monitoring results. The samples collected for *E. coli* analysis during the modelled period focused on the last 4 of the 5 high flow events. To provide an *E. coli* input into the model ($C1_{in}$) we calculated the average measured concentration for each event (Table 1). No samples were collected during Event 1, therefore we used the average *E. coli* concentrations from Event 2 as a best estimate and to allow the model to spin up. Additionally, base-flow samples were not targeted during this monitoring period and therefore samples associated with base-flow prior to the rising limbs were very limited. We, therefore, used the average of all base-flow data collected from 2001 to 2005. The event average concentrations were applied from the start of the high-flow event until the flow rate fell below 0.5 L s^{-1} and then switched to the base-flow concentration until the next event. The averaged outflow *E. coli* concentration were used to compare with the modelled outputs.

3. RESULTS AND DISCUSSION

The wetland had stopped flowing in the summer of 2005/2006 (Southern Hemisphere). In April 2006 there was a large rain event which filled the wetland, and it then continued to flow until late September, during which time there were 5 high flow events (Figure 1). The *E. coli* concentrations across the 4 measured events were highly variable but clearly showed the higher concentrations measured in the outflows from the wetland. The averaged model input concentrations appear to realistically reflect the changes in the measured concentrations between events. Interestingly the averaged inflow *E. coli* concentration measured for Event 3 (37 MPN/100mL) was lower than the long-term measured baseflow average concentration (53 MPN/100mL) so the modelled input concentration decreased for Event 3 (Table 1, Figure 1). However, the average measured outflow concentration from Event 3 was higher than the background concentration (Figure 1). The measured inflow and outflow concentrations for Event 5 were much higher than for the other events and may reflect recent grazing or farm dairy effluent irrigation in the catchment (Stott et al. 2023).

Table 1. Average *E. coli* concentrations (MPN 100mL^{-1}) measured in inflow to Cell 1 and outflow from Cell 2 of the Toenepi constructed wetland system. The averaged input concentrations were used as an input in the model simulations and the averaged outputs were used to compare with the model outputs.

Time period	*E. coli* in [Ec_{in}]	*E. coli* out
Base flow*	53	315
Event 1+	294	1035
Event 2	294	1035
Event 3	37	438
Event 4	141	1220
Event 5	7093	11,121

* Concentrations based on base-flow samples collected during 2001–2005.
+ Concentrations assumed to be the same as the measured data from Event 2.

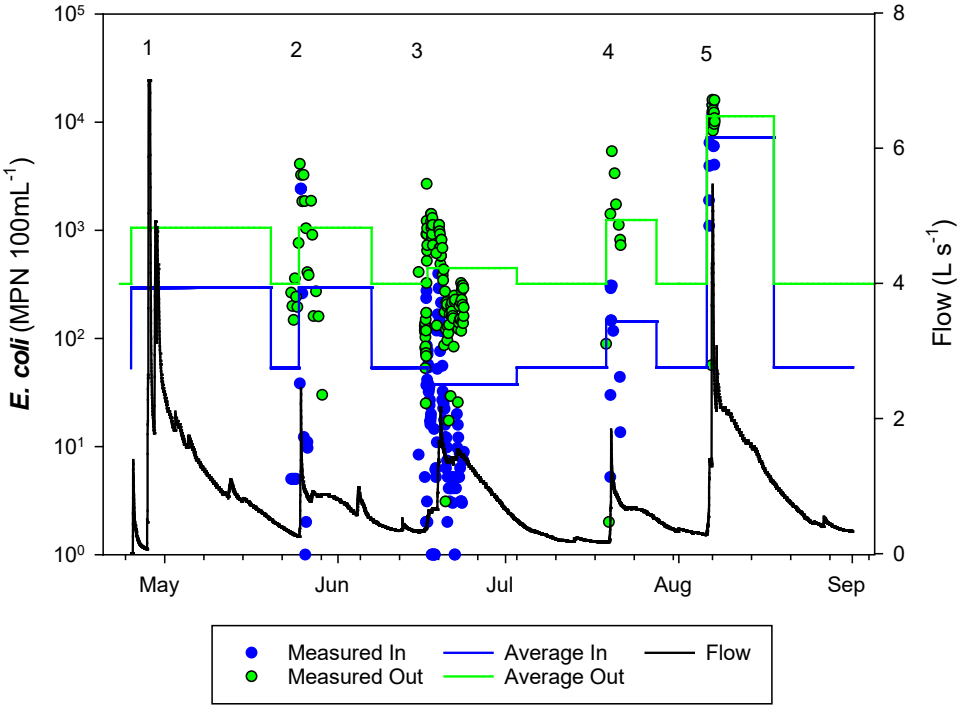

Figure 1. Measured inflow rate into the Toenepi wetland for the 5 events during this winter period. Measured *E. coli* in the inflow and outflow of the wetland and the averaged *E. coli* concentrations from Table 1 used in the modelling.

When the wetland model was run using a negative *k* value (to represent typical first order die-off of *E. coli* in a water environment) modelled outflow concentrations never exceeded modelled inflow concentrations and hence cannot explain the consistently higher concentrations of *E. coli* measured in the outflow (data not shown). From the mesocosm experiments carried out in the Toenepi wetlands, the mean and maximum growth rate observed were 0.1 and 0.2 *ln* day^{-1}, respectively (A. Cookson, pers. comm.). When these growth rates were used in the model, the outflow *E. coli* concentrations did not match the average outflow concentrations (Figure 2). Setting the *k* value to 0.3 provided the best match of modelled outflow to average outflow concentrations (Figure 2). In this scenario the modelled *E. coli* concentrations in the outflow underestimated the average concentration for Event 2, overestimated Event 3 and peaked at the averaged concentration for Events 4 and 5 (Figure 2). For the model to generate *E. coli* outflow concentrations similar to the measured concentrations we need to invoke a growth rate in the wetland water column that is greater than any growth rate measured in the microcosm and mesocosm experiments (A. Cookson, pers. comm.). Higher *E. coli* growth rates have been observed in a study conducted on a concrete lined stream with high temperatures and high nutrient inputs (Surbeck et al. 2010). In the Toenepi wetland environment, organic C and nutrient availability

will be high from decomposing plant litter although emergent plants will compete for nutrients during the cold winter drainage period (Tanner et al. 2005). Therefore, we believe that the measured *E. coli* water growth rates in our study to be more realistic of the situation in a wetland (A. Cookson, pers. comm.).

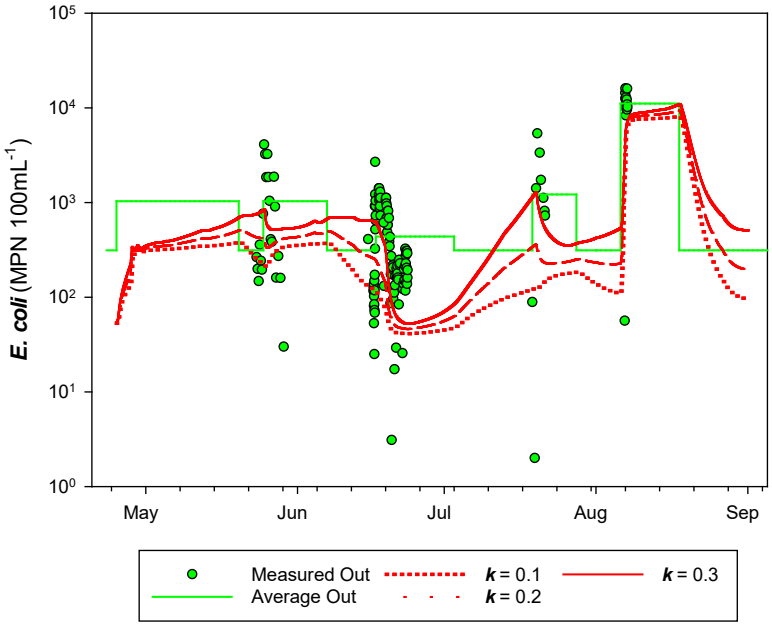

Figure 2. Measured and averaged *E. coli* concentrations in the wetland outlet compared to the modelled outputs using different growth rates

The dynamics of the measured and modelled *E. coli* concentrations were investigated in more detail at the event-scale. If we assume that *E. coli* can grow in the wetland water column then we would expect this to predominantly occur during the base-flow periods (when there is minimal water flushing through the wetlands) and this is what the model shows. For Events 2, 3 and 4, the modelled *E. coli* concentrations steadily increase during the low flow period leading up to an event due to the modelled growth of *E. coli* in the wetland (Figure 2). Then, at the start of Events 2, 3 and 4, the modelled *E. coli* concentration in the outlet decreases as the wetland water is effectively diluted with low *E. coli* concentrations in the inflow. However, for Event 5, the modelled baseflow outflow concentration was still less than 1000 *E. coli* 100mL^{-1} at the beginning of the event and therefore, the modelled inflow of high flows with 7093 *E. coli* 100mL^{-1} rapidly increased the concentration in the wetlands and hence in the modelled outflow (Figures 2 and 3). The modelled outflow concentrations then rapidly increased during the high flow period of Event 5 to match the average measured outflows at the end of the high flow period before rapidly decreasing as the model switched to low concentration *E. coli* inflows during the baseflow (Figure 2). This temporal pattern is expected given that the model is assuming growth of *E. coli* in the water column. To determine if these modelled dynamics are realistic, we focused on the measured data for each of the 4 measured events (Figure 3). The measured data clearly shows that the concentrations of *E. coli* in the outflow just prior to each high flow event are not the elevated numbers predicted by the water column growth model. The outlet *E. coli* concentrations increase dramatically at the beginning of the high flow periods and often even before the flow rate in the wetland increased.

The microcosm and mesocosm experiments were conducted in the water phase of the wetland and so the model described here only simulates *E. coli* concentrations for the water phase of the Toenepi wetland. Other potential sources of *E. coli* within the wetland exist such as biofilms attached to the plant surfaces in the wetland (Mulling et al. 2013). No measurements were made of *E. coli* in the biofilms of the wetlands nor growth studies of *E. coli* in biofilms in the wetland. However, from the timing of the wetland measurements, it can be seen that the concentration in the outlet tended to increase dramatically as soon as the outlet flow started to increase (Figure 3). The rapid increase occurs long before the fresh inputs to the first cell could reach the outlet of the second cell of the wetland (data not shown). This rapid increase on the rising limb of the flows is consistent with the mechanism of disturbing an *in situ* storage reservoir of *E. coli* present in a flowing stream (Muirhead et al. 2004). Another potential reservoir of *E. coli* in a wetland could be in the sediments. Wetlands are designed to slow water flow to allow the treatment effect (Shukla et al. 2021) and settlement of faecal microbes bound

to particles following which they may persist in wetland sediments for extended periods (Stenström and Carlander, 2001). Adsorption of faecal microbes to surfaces is also considered an important removal mechanism in wetlands (Stott and Tanner 2005). Therefore, the rapid increases of *E. coli* on the rising limb of an event may be due to resuspension from wetland sediments, or more likely, disturbance from the biofilms attached to plants and leaf litter in the water column. Wetland plants are emergent and therefore biofilms could be physically disturbed by wind or rain drop impact before a drainage events start. This hypothesis of a biofilm source for the high export of *E. coli* from the Toenepi wetland would be a fruitful area for further research into this unique observation of the net export of *E. coli* from the Toenepi wetland.

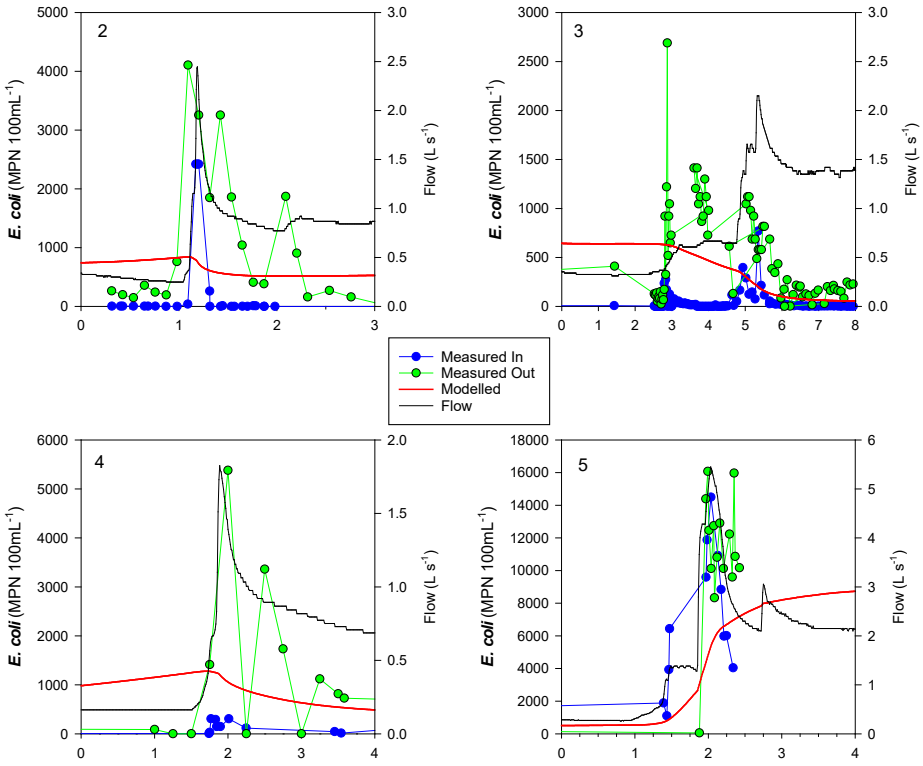

Figure 3. Data for individual events monitored during 2006. Shown are measured flow rates, measured *E. coli* concentrations and modelled outputs ($k = 0.3$) for Events 2 to 5. The X axis scale shows days during the individual events.

4. CONCLUSIONS

A model of *E. coli* growth in a constructed wetland was developed to test the hypothesis that the unusual net export of *E. coli* from the Toenepi wetland was caused by growth of *E. coli* in the water column. The model was driven by recently measured growth rates of both faecal and potentially naturalized *Escherichia* strains and was tested against historical measured flow rate data and *E. coli* concentrations measured into and out of the Toenepi wetland. The model demonstrated that growth in the water column could not explain the dynamics of the system or the net export of *E. coli* from the Toenepi wetland for several reasons. Firstly, *E. coli* growth rates in the water column would have to be consistently greater than any measured growth rate for the model outputs to match the average *E. coli* concentrations measured at the wetland outlet. Secondly, the growth model predicted that the *E. coli* concentrations in the outlet should steadily increase during low flow periods between rainfall driven drainage events. However, measured data clearly showed *E. coli* concentrations remaining low during low flow conditions and increasing dramatically at the beginning of each high flow event. This modelling analysis indicates that the hypothesis that, the unusual net export of *E. coli* from the Toenepi wetland is caused solely by growth of *E. coli* in the water column, should be rejected. Instead, the dynamics of the *E. coli* concentrations at the wetland outlet indicate alternative sources of *E. coli* are likely contributing to *E. coli* concentration in the water column such as *E. coli* associated with biofilms attached to plants and leaf litter in the wetland.

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